# Sequence Requirement for Trimethylation of Yeast Cytochrome *c*†

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ABSTRACT: Lysine 72 (using the the vertebrate numbering system) is trimethylated in cytochromes *c* from fungi and plants but not from higher animals. We have investigated the characteristics of an amino acid sequence required for trimethylation of lysine 72 by examining 21 altered iso-1-cytochromes *c* from *Saccharomyces cerevisiae* having single replacements in the region encompassing residues 67 through 77. These results indicated that tyrosine 74 is critical for trimethylation of lysine 72, whereas replacements at other positions did not produce significant diminutions. Various replacements of tyrosine 74 resulted in different levels of inhibition, with the Y74F replacement causing no significant reduction, and the Y74E and Y74K replacements completely or almost completely preventing trimethylation of lysine 72. However, other similarly spaced lysine and tyrosine residues at other sites in the protein did not result in trimethylation of the lysine residue. Thus, a properly situated aromatic residue, determined by the overall conformation of apocytochrome *c* in the vicinity of lysine 72, appears to be essential for trimethylation.

Specific lysine residues are methylated in cytochromes *c* from certain eukaryotes but not from any prokaryotes. Wheat germ cytochrome *c* (Delange et al., 1969) and cytochromes *c* from other plants (Brown & Boulter, 1973; Ramshaw & Boulter, 1975; Brown et al., 1973) contain *N*-ϵ-trimethyllysine (Tml)[1] at positions 72 and 86, except for *Enteromorpha intestinalis* (Meatyard & Boulter, 1974), which is trimethylated only at Lys72 (using the vertebrate cytochrome *c* numbering system). Many fungi contain Tml at the single position 72, including iso-1-cytochrome *c* and iso-2-cytochrome *c* (DeLange et al., 1970) from *Saccharomyces cerevisiae*, and cytochromes *c* from *Neurospora crassa* (DeLange et al., 1969), *Debaromyces kloeckeri* (Sugeno et al., 1971), and *Candida krusei* (DeLange et al., 1970) but not from *Ustilago sphaerogena* (Bitar et al., 1972). *Humicola languinosa* cytochrome *c* contains *N*-ϵ-trimethyllysine at position 86 and *N*-ϵ-dimethyllysine at position 72 (Morgan et al., 1972). *Hansenula anomala* cytochrome *c* contains *N*-ϵ-trimethyllysine at positions 72 and 73, and *N*-ϵ-dimethyllysine and *N*-ϵ-monomethyllysine at position 55 (Becam & Leder, 1981). *Crithidia oncopelti* cytochrome *c*-557 contains *N*-ϵ-trimethyllysine at positions 72 and −8 and *N*-α-dimethylproline at the N-terminal position −10 (Pettigrew et al., 1975; Smith & Pettigrew, 1980). *Euglena gracillus* cytochrome *c*-558 contains *N*-ϵ-trimethyllysine at positions 86 (Pettigrew, 1973; Lin et al., 1973). No methylated lysine residues were found after specific examinations of cytochromes *c* from vertebrates and invertebrates, including lamprey, dogfish, bullfrog, turtle, rattlesnake, turkey, mammals, and *Samia cynthia* (Delange et al., 1970).

Cytochrome *c* specific protein lysine methyltransferases (*S*-adenosylmethionine:cytochrome *c*-lysine *N*-methyl transferase) have been partially purified and characterized from *N. crassa* (Durban et al., 1978), *S. cerevisiae* (DiMaria et al., 1979), and wheat germ (Nochumson et al., 1977). These methyltransferases exhibit high specificity toward cytochrome *c* as a substrate and toward particular amino acid residues in the substrate protein (Nochumson et al., 1977). In addition, holocytochrome *c* was found to be a poorer substrate than apocytochrome *c*, which was prepared either from holocytochrome *c* or by translation *in vitro* (DiMaria et al., 1979; Park et al., 1987). However, both apocytochrome *c* and holocytochrome *c* lost their substrate capability when bound to mitochondria in low ionic conditions, but this capability was restored when the cytochromes *c* were released from the mitochondria by KCl treatment. Also cycloheximide inhibited both protein synthesis and methylation (Farooqui et al., 1980). These results, and the fact that the methylase is located in the cytosol and not mitochondria, suggest that nascent apocytochrome *c* was methylated *in vivo*, either co- or post-translationally, before being associated with mitochondria (DiMaria et al., 1979; Park et al., 1987).

Furthermore, the activity of the methylase is lower in extracts of yeast grown under conditions of catabolite repression or anaerobiosis, the same conditions where cytochrome *c* is low. Also, during anaerobic to aerobic adaptation, the methylase was induced with cytochrome *c*, indicating that the synthesis of cytochrome *c* and the methylase are at least partially coordinately regulated (Liao & Sherman, 1979). These results suggest that cytochrome *c* may be the natural and sole substrate.

The biological function and importance of cytochrome *c* methylation have not been clearly established. The results of some experiments performed *in vitro* led to the suggestion that Tml72 is required for mitochondrial import of cytochrome *c* in yeast (Polastro et al., 1977, 1978a,b; Park et

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[1] Abbreviations: Tml, *N*-ϵ-trimethyllysine; Tml72, Tml at amino acid position 72 of cytochrome *c*, using the vertebrate numbering system, or at position 77, using the iso-1-cytochrome *c* numbering system.

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Table 1: Altered Forms of Iso-1-cytochrome *c* Used to Examine *N*-$\epsilon$-Trimethylation of Lysine 72[a]

| replacement[b] | allele | strain number | % iso-1 | % Tml | reference for replacements |
|---|---|---|---|---|---|
| none | *CYC1*$^+$ | Various | 100 | 100 | – |
| Y67F[c] | *CYC1-991* | B-8083 | 100 | 100 | this investigation |
| L68I[c] | *CYC1-992* | B-8084 | 100 | 100 | this investigation |
| T69E[c] | *CYC1-993* | B-8085 | 100 | 100 | this investigation |
| N70E[c] | *CYC1-994* | B-8086 | 100 | 100 | this investigation |
| P71E | *CYC1-1072* | B-8238 | 100 | 100 | this investigation |
| P71I[c] | *CYC1-24-F* | B-1499 | 100 | 100 | Ernst et al., 1985 |
| P71V[c] | *CYC1-104-B* | B-1508 | 100 | 80 | Ernst et al., 1985 |
| P71T[c] | *CYC1-24-N* | B-1613 | 100 | 100 | Ernst et al., 1985 |
| P71S[c] | *CYC1-24-L* | B-1611 | 100 | 100 | Ernst et al., 1985 |
| K72R[c] | *CYC1-784* | B-6765 | 100 | 0 | Holzchu et al., 1987 |
| K72D[c] | *CYC1-785* | B-6842 | 100 | 0 | T. Cardillo and F. Sherman, unpublished |
| K73E | *CYC1-1074* | B-8240 | 90 | 100 | this investigation |
| Y74E[c] | *CYCl-995* | B-8087 | 80 | 0 | this investigation |
| Y74A | *CYC1-1073* | B-8239 | 90 | 50 | this investigation |
| Y74K[c] | *CYC1-94-N* | B-2068 | 100 | 0 | K. Mann and F. Sherman, unpublished |
| Y74Q | *CYC1-94-O* | B-3004 | 100 | 30 | K. Mann and F. Sherman, unpublished |
| Y74F | *CYC1-1075* | B-8241 | 100 | 75 | this investigation |
| I75Q[c] | *CYC1-1149* | B-8359 | 40 | 90 | this investigation |
| I75Y | *CYC1-1150* | B-8371 | 30 | n.d.[d] | this investigation |
| I75R | *CYC1-1147* | B-8338 | 25 | n.d. | this investigation |
| I75D | *CYC1-1148* | B-8339 | 10 | n.d. | this investigation |
| I75L | *CYC1-1146* | B-8337 | 90 | n.d. | this investigation |
| P75E[c] | *CYC1-997* | B-8089 | 90 | 100 | this investigation |
| P76L[c] | *CYC1-94-J* | B-1825 | 90 | 100 | Hampsey et al., 1988 |
| G77E[c] | *CYC1-998* | B-8090 | 100 | 100 | this investigation |
| K72R I75Y | *CYC1-1151* | B-8372 | 30 | 0 | this investigation |
| P71K K73Y Y74I | *CYC1-1155* | B-8455 | 45 | 0 | this investigation |
| A[−1]K G1Y | *CYC1-1159* | B-8459 | 90 | 0[e] | this investigation |
| G6Y | *CYC1-1160* | B-8460 | 100 | 0[e] | this investigation |
| rat | *CYC1-RAT1* | B-7667 | 40 | 80 | Clements et al., 1989 |
| rat | *CYC1-RAT2* | B-7671 | 80 | 80 | Clements et al., 1989 |
| pigeon | *CYC1-PIGEON1* | B-7835 | 40 | 75 | Hickey et al., 1991 |
| pigeon | *CYC1-PIGEON2* | B-7836 | 100 | 65 | Hickey et al., 1991 |
| pigeon | *CYC1-PIGEON3* | B-7837 | 150 | 65 | Hickey et al., 1991 |

[a] Altered iso-1-cytochromes *c* were obtained from the following: intragenic revertants of *cyc1* mutants (*CYC1-24-F*, *CYC1-104-B*, etc.); mutants obtained by oligonucleotide-directed mutagenesis (*CYC1-784* and *CYC1-785*); or mutants obtained by transforming *cyc1-812* or *cyc1-31* directly with synthetic oligonucleotides as indicated by "this investigation". A pseudogene, *CYC1-RAT*, or a synthetic gene, *CYC1-PIGEON*, was used to obtain vertebrate cytochromes *c* from yeast. [b] Replacement established by protein analysis and in some cases confirmed by DNA sequencing. [c] Replacement confirmed by DNA sequencing. [d] ND, not determined. [e] Tml residues in the amino-terminal region (see Figure 2).

al., 1987), a conclusion that was later refuted by Cessay et al. (1991). On the other hand, pulse-chase experiments suggested that Tml72 protected cytochrome *c* from proteolytic degradation *in vivo* (Farooqui et al., 1981). At the end of a 40 h chase period, the extent of degradation of the unmethylated form was three times higer than the methylated form. However, cycloleucine, used to inhibit cytochrome *c* methylation, could have also affected the protein degradation system.

Cessay et al. (1994) also investigated the turnover of iso-1-cytochrome *c* having amino acid replacements at positions 71−74 and, consequently, different levels of trimethylation of Lys72. Although the K72R iso-1-cytochrome *c* had the shortest half-life, 9 h compared to 23 h for the normal protein, there was no significant correlation between the half-life and the degree of trimethylation of Lys72 among the six other iso-1-cytochromes *c* having various replacements.

The function of trimethylated Lys72 has been investigated in yeast by examining the consequences of K72R (Holzschu et al., 1987) and K72D (T. Cardillo and F. Sherman, unpublished results) replacements. Spectroscopic measurements revealed that K72R and K72D iso-1-cytochromes *c* were at normal or near normal levels (Table 1). Furthermore, growth in lactate medium indicated that the K72R iso-1-cytochrome *c* had normal or near-normal activity *in vivo* (Holzschu et al., 1987). On one hand, the maintenance of Lys72 in 96 different species, except *Tetrahymena pyriformis* (Moore & Pettigrew, 1990), and the maintenance of the apparently specific methylase in numerous plant and fungal species indicate that Lys72 or Tml72 is essential from an evolutionary point of view. On the other hand, the results with the K72R and K72D iso-1-cytochromes *c* indicate that a lysine or trimethylated lysine at position 72 is not absolutely required for biosynthesis, mitochondrial import, or activity, and the residue at this position is not critical. It should be noted that conserved residues at other sites in iso-1-cytochrome *c* also can be replaced without drastically effecting its biosynthesis or function (Hampsey et al., 1988). Because Lys72 is at most only marginally critical for function, the role of methylation may be difficult to assess, especially from *in vitro* studies, and the biological function of trimethylation remains to be elucidated.

Cessay et al. (1994) reported various degrees of diminished trimethylation of Lys72 in seven iso-1-cytochromes *c* having amino acid replacements at positions 71−74. However, no clear conclusions were presented concerning the importance of residues at specific positions except that the "methyltransferase displayed a very strict primary sequence requirement adjacent to the methylation site for proper substrate recognition" (see Results and Discussion).

We report herein the the characteristics of an amino acid sequence required for trimethylation of Lys72. The degree

of trimethylation of Lys72 was determined in iso-1-cytochromes *c* having single amino acid replacements in the region encompassing position 72. The results indicate that an aromatic residue, tyrosine or phenylalanine, is required at at position 74 for optimal trimethylation of Lys72, whereas replacements at other nearby sites were less critical. Because lysine residues were not trimethylated when situated at other sites and still having properly placed tyrosine residues, we believe that an overall conformation of apo-cytochrome *c* in the vicinity of Lys72 is also essential for trimethylation.

## EXPERIMENTAL PROCEDURES

*Genetic Nomenclature and Yeast Strains*. *CYC1*$^+$ denotes the wild-type allele encoding iso-1-cytochrome *c* in the yeast *S. cerevisiae*, whereas *CYC1* denotes the locus and is the generic symbol of any allele. The *cyc1-31* and *cyc1-812* alleles cause a complete deficiency of iso-1-cytochrome *c*, whereas *CYC1* mutants listed in Table 1 have at least a partially functional iso-1-cytochrome *c*. *CYC7*$^+$ denotes the wild-type allele encoding iso-2-cytochrome *c*, and *cyc7-67* denotes a partial deletion of the *CYC7* locus that results in the complete deficiency of iso-2-cytochrome *c*.

The yeast strains, listed in Table 1, were either chosen from our collection as cited, or were made specifically for this study by transforming strains directly with the synthetic oligonucleotides as described below. Thus, the strains represent three isogenic series; the intragenic revertants were derived from D311-3A; the mutants obtained by oligonucleotide-directed mutagenesis were derived from B-6748; and the mutants derived by transforming strains directly with synthetic oligonucleotides were derived from B-7528.

*Numbering Amino Acid Positions in Cytochromes c*. The numbering of amino acid positions used in this paper is based on vertebrate cytochrome *c*, in which, for example, Lys72 corresponds to position 77 of iso-1-cytochrome *c* (see Figures 1 and 2).

*Oligonucleotide Transformation*. Strains with specific alterations were made for this study by transforming strain B-8079 (*MAT***a** *cyc1-812 cyc7-67 ura3-52 lys5-10*) (Yamamoto et al., 1992a) or B-7528 (*MAT***a** *cyc1-31 cyc7-67 ura3-52 lys5-10*) (Moerschell et al., 1988) directly with the synthetic oligonucleotides listed in Table 2 and using the procedures described by Yamamoto et al. (1992b). The oligonucleotides were used for oligonucleotide-directed mutagenesis (Table 2) were synthesized on an Applied Biosystems 380A DNA synthesizer and prepared as previously described by Moerschell et al. (1988). The alterations were confirmed by DNA sequencing of appropriate regions that were amplified by the PCR or by amino acid compositional analysis of the peptides encompassing the replacement.

*Determination of Cytochrome c Content*. Total amounts of cytochrome *c* were determined by spectroscopy examination of intact cells at −196 °C (Sherman & Slonimski, 1964) and by comparing the intensities of the $C_\alpha$ bands at 547 nm to the $C_\alpha$ bands of strains having known amounts of cytochrome *c*. A more accurate determination of cytochrome *c* content in intact cells was made by low temperature (−196 °C) spectra recording with an Aviv model 14 spectrophotometer (Hickey et al., 1991).

*N-ϵ-Trimethylation Analysis*. Altered iso-1-cytochromes *c* were isolated as described previously (Sherman et al., 1968) and further purified by HPLC on a μBondasphere column

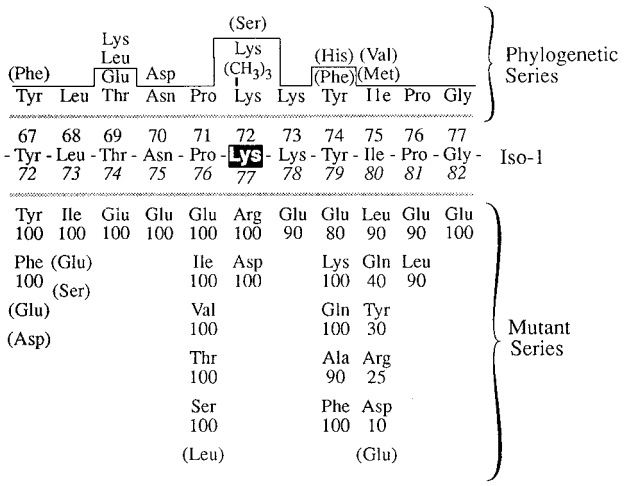


FIGURE 1: Phylogenetic and mutant series. The normal amino acid sequence of iso-1-cytochrome *c* is shown in the middle of the figure, with the vertebrate and the yeast numbering system presented, respectively, above and below the sequence. The composite of 86 different eukaryotic cytochromes *c* from 96 different eukaryotic species (Moore & Pettigrew, 1990) is present at the top of the figure. The following amino acid residues from the following species containing unusual cytochromes *c* are shown in parenthesis: Ser72 and His74, *T. pyriformis*; Phe67 and Val75, *T. pyriformis* and *E. gracilis*; Met75, *C. oncopelti* and *C. fasciculata*; and Phe74, *C. oncopelti*, *C. fasciculata*, and the fungus *H. languinosa*. The residue found in *at least* fungal cytochromes *c*, and in most eukaryotic cytochromes *c*, except for those at position 69, are presented above the iso-1-cytochrome *c* sequence, below the broken line. The residues above the broken line are found in cytochromes *c* from species other than fungi. For example, Glu69 is found in cytochrome *c* from most animal and fungal species, but not in iso-1-cytochrome *c* or iso-2-cytochrome *c*, and Leu69 is found in cytochrome *c* from higher plants. The mutant series, shown at the bottom of the figure, represents amino acid replacements in iso-1-cytochromes *c* uncovered in this and other studies (Table 1). The levels of the functional altered forms, as percentages of the normal level of iso-1-cytochrome *c*, are shown below each of the replacements. The replacements of nonfunctional forms are shown in parenthesis. These nonfunctional forms were identified as *cyc1* missense mutations (Hampsey et al., 1986) or were deduced because of the lack of recovery of transformants (see text).

(3.9 × 250 mm, C4, 15 μm, 300 Å, Waters Associates), using a linear gradient elution of 0−80% 2-propyl alcohol/acetonitrile (7/3, v/v) in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min for 40 min. A total of 10 nmol of each cytochrome *c* was digested with *Achromobacter* protease I (lysylendopeptidase, Wako Pure Chemicals) at an enzyme to substrate ratio of 1/400 (mol/mol) in 100 μL of 100 mM Tris-HCl buffer (pH 9.0) at 37 °C for 6 h. The resulting peptides were separated by HPLC on a μBondasphere column (3.9 × 150 mm, C18, 100 Å, Waters Associate), using a linear gradient of 0−40% 2-propyl alcohol/acetonitrile (7/3, v/v) in 0.1% trifluoroacetic acid at flow rate of 0.8 mL/min for 40 min. The normal and altered peptides corresponding to Asn56-Lys73 were identified by comparison of their HPLC maps with that from wild-type yeast iso-1-cytochrome *c*, followed by analyses for their amino acid compositions. The degree of N-ϵ-trimethylation of a series of altered iso-1-cytochrome *c* were estimated based on their Tml contents and recovery of peptides. Also amino acid substitution in mutant proteins was comfirmed by amino acid analysis of peptide derived from each protein. The amino acid analyses were carried out with a PICO-TAG system (Waters Associates). Peptides and proteins were hydrolyzed *in vacuo* for 24 h at 110 °C in 6 N HCl, the amino acids

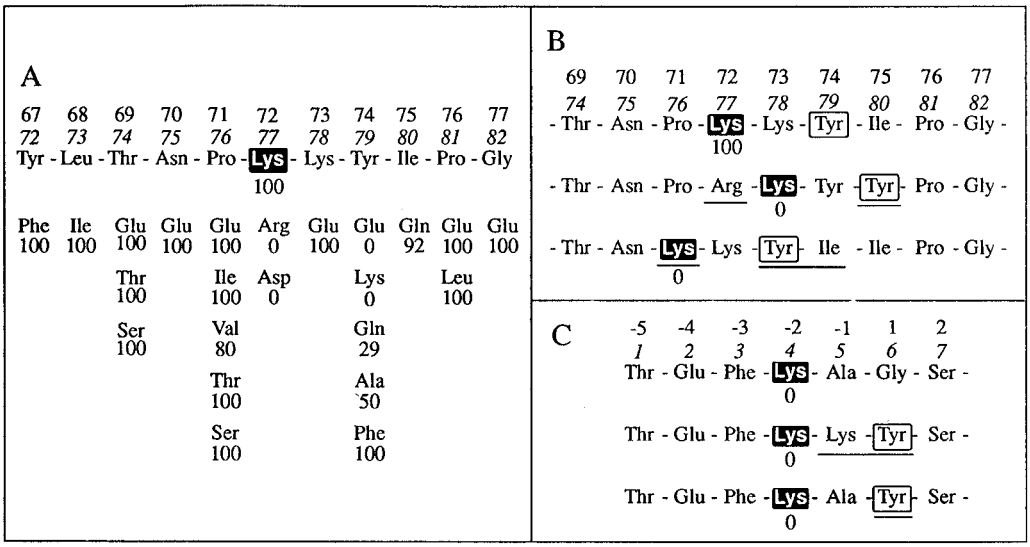


FIGURE 2: Proportion of Tml residues in altered forms of iso-1-cytochrome *c*. The amino acid sequence of normal iso-1-cytochrome *c* is shown in the top line of each panel. Percentages of Tml72, presented in the figure, are taken from column 5 of Table 1. (A) Percentages of Tml72 are presented below each amino acid replacement. (B, C) Percentages of trimethylation of the lysine residues highlighted in black are presented below each of these residues. Abnormal residues are underlined. The tyrosine residues situated two positions to the right of the lysine residues are shown in open boxes. The amino acid positions of iso-1-cytochrome *c* are denoted both with the vertebrate numbering system and with the iso-1-cytochrome *c* numbering system (*italics*).

were coupled with PITC, and the resulting PTC-amino acids were analyzed by HPLC on a PICO-TAG column (Waters Associates). Examples of HPLC profiles of peptides obtained by lysylendopeptidase digestion of normal and mutant forms of iso-1-cytochrome *c* are presented in Figure 3.

## RESULTS AND DISCUSSION

*Iso-1-cytochrome c with Amino Acid Replacements at the 67−77 Sites*. The amino acid requirements for methylation of Lys72 were investigated with 21 altered iso-1-cytochromes *c* having single amino acid replacements within positions 67 through 77 (Table 1, Figure 2A). The *CYC1* mutants, which were either choosen from our collection, or were specifically constructed for this study, represents the following three isogenic series: those indirectly derived from D311-3A as intragenic revertants; *CYC1-784* and *CYC1-785* derived from B-6748 by oligonucleotide-directed mutagensis; and those directly or indirectly derived from B-7528 by transformation with oligonucleotides. For example, the *CYC1-94-N*, *CYC1-94-O*, and *CYC1-94-J* intragenic revertants were derived from the *cyc1-94* mutant, which contains a TAA nonsense mutation corresponding to amino acid position 74. The majority of the mutants were specifically prepared by the convenient procedure of transforming the strain *cyc1-812* with the oligonucleotides listed in Table 2. The *cyc1-812* allele contains a deletion of a single A·T base pair, resulting in a nonsense and a frameshift mutation corresponding to position 74. Transformation with the oligonucleotides corrected the nonsense/frameshift mutation and produced the desired amino acid replacement. The recovery of amino acid replacements at all of the 67−77 positions illustrated the power of this method. Furthermore, correct sequences were recovered after examining only one transformant in 15 out of 16 mutants; only one mutant required the examination of two transformants, and in this case the nonsense/frameshift mutation and the mutation producing the desired amino acid replacement were separated by 20 nucleotides (Table 2). In addition, this procedure results in strains with a single copy of the altered gene at the normal chromosomal position.

Attempts were made to obtain glutamic acid replacements at each of the sites, because glutamic acid differs drastically from all of the residues in this conserved region. Such glutamic acid replacements were recovered at all of the 11 sites except at positions 72, 73, and 80. Because recovery of an amino acid replacement transformation with an oligonucleotide requires that the iso-1-cytochrome *c* be at least partially functional, the lack of recovery of a particular replacement could be due to the lack of function of the iso-1-cytochrome *c*. However, only a minimal degree of function is required, and mutants having as low as 1% of the normal activity have been recovered with this method. Thus, glutamic acid at position 67, 68, or 75 may not be compatible with function. Nevertheless, I75Q, I75Y, I75R, I75D, and I75L replacements were obtained by transformation with specific synthetic oligonucleotides.

*Levels and Function of the Mutationally Altered Iso-1-cytochromes c*. The total amounts of the mutationally altered iso-1-cytochromes *c* in intact cells were estimated from the heights of the $C_\alpha$ band by spectroscopic examination. The results presented in Table 1 and Figure 1 revealed that all of the altered iso-1-cytochromes *c* were at normal or near-normal levels except those having replacements at position 75. For example, the I75L iso-1-cytochrome *c* was slightly below the normal level, whereas the I75D iso-1-cytochrome *c* was only at approximately 10% of the normal level.

Although we have not investigated quantitative growth curves in lactate medium for estimating the level of function of the iso-1-cytochromes *c*, these altered forms were at least partially functional as indicated by the growth of the *CYC7*$^+$ revertants on lactate medium, and the *cyc7-67* transformants on glycerol medium. Also presented in Figure 1 are the nonfunctional forms that were identified as *cyc1* missense mutations (Y67D, L68S, P71L [Hampsey et al., 1988]), or that were deduced because of the lack of recovery of transformants (Y67E, L68E, and I75E).

Table 2: Oligonucleotides Used to Produce *CYC1* Mutants by Transformation

| allele | parental allele | oligo. no. | sequence[a] | recovered[b] |
|---|---|---|---|---|
| *CYC1-991* | *cyc1-812* | OL90.085 | TGTCAGAGTTCTTGACTAACCCAAAGAAATATATTCCTGG | 1/2 |
| *CYC1-992* | *cyc1-812* | OL90.087 | GTCAGAGTACATTACTAACCCAAAGAAATATATTCCTGGT | 1/1 |
| *CYC1-993* | *cyc1-812* | OL90.088 | GTCAGAGTACTTGGAAAACCCAAAGAAATATATTCCTGGT | 1/1 |
| *CYC1-994* | *cyc1-812* | OL90.089 | AGAGTACTTGACTGAACCAAAGAAATATATTCCTGGTACC | 1/1 |
| *CYC1-995* | *cyc1-812* | OL90.093 | CTTGACTAACCCAAAGAAAGAAATTCCTGGTACCAAGATG | 1/1 |
| *CYC1-997* | *cyc1-812* | OL90.095 | CTAACCCAAAGAAATATATTGAAGGTACCAAGATGGCCTT | 1/1 |
| *CYC1-998* | *cyc1-812* | OL90.096 | AACCCAAAGAAATATATTCCTGAAACCAAGATGGCCTTTG | 1/1 |
| *CYC1-1072* | *cyc1-812* | OL91.230 | GTCAGAGTACTTGACTAACGAGAAGAAATATATTCCTGGT | 1/1 |
| *CYC1-1073* | *cyc1-812* | OL91.231 | GTACTTGACTAACCCAAAGGAATATATTCCTGGTACCAAG | 1/1 |
| *CYC1-1074* | *cyc1-812* | OL91.232 | TTGACTAACCCAAAGAAAGCTATTCCTGGTACCAAGATGG | 1/1 |
| *CYC1-1075* | *cyc1-812* | OL91.233 | TTGACTAACCCAAAGAAATTCATTCCTGGTACCAAGATGG | 1/1 |
| *CYC1-1146* | *cyc1-812* | OL91.311 | GACTAACCCAAAGAAATATTTGCCTGGTACCAAGATGGCC | n.d. |
| *CYC1-1147* | *cyc1-812* | OL91.312 | GACTAACCCAAAGAAATATAGACCTGGTACCAAGATGGCC | n.d. |
| *CYC1-1149* | *cyc1-812* | OL91.310 | GACTAACCCAAAGAAATATCAACCTGGTACCAAGATGGCC | 1/1 |
| *CYC1-1150* | *cyc1-812* | OL92.013 | GACTAACCCAAAGAAATATTACCCTGGTACCAAGATGGCC | n.d. |
| *CYC1-1151* | *cyc1-812* | OL92.015 | CTTGACTAACCCAAGAAAATATTACCCTGGTACCAAGATG | 1/1 |
| *CYC1-1155* | *cyc1-812* | OL92.069 | AGTACTTGACTAACAAAAAGTACATTATTCCTGGTACCAA | 1/1 |
| *CYC1-1159* | *cyc1-31* | OL92.104 | AATAATGACTGAATTCAAGAAGTACTCTGCTAAGAAAGGT | 1/1 |
| *CYC1-1160* | *cyc1-31* | OL92.105 | AATAATGACTGAATTCAAGGCCTACTCTGCTAAGAAAGGT | 1/1 |

[a] The nucleotides correcting the nonsense/frameshift mutations of the *cyc1-812* and *cyc1-31* mutations are double underlined, and these correspond, respectively, to amino acid positions 79 and 4. The nucleotides encoding the desired amino acid replacements are underlined. [b] The number of transformants containing the expected sequence, over the number of transformants examined. n.d., not determined.

Mulligan-Pullyblank et al. (1996) have reported 116 multiple amino acid replacements of residues encompassing positions 74−79 of iso-1-cytochrome *c*. However, their results and the results presented in Figure 1 are difficult to compare because they did not report the levels or degree of function of the iso-1-cytochromes *c*. Furthermore, two adjacent residues at positions 74 and 75, positions 76 and 77, and positions 78 and 79 were concomitantly replaced. Although the authors stressed the relative occurrence of the multiple replacements, there is no reason to believe that the frequencies reflect the degree of function. On the other hand, Herrmann et al. (1996) reported the functionality *in vivo* of iso-1-cytochromes *c* having all amino acid replacements at position 73. Only strains having K73C iso-1-cytochrome *c* exhibited greatly diminished growth rates in media containing nonfermentable carbon sources, indicating that the functional requirement at position 73 can be met by a variety of amino acids.

In addition to summarizing the replacements of the iso-1-cytochromes *c* in Figure 2, we have presented a composite of 86 different eukaryotic cytochromes *c* from 96 different eukaryotic species. Excluding cytochrome *c* from *Tetrahymena pyriformis*, the residues at all sites are highly conserved in the phylogentic series except for position 69. The other sites contain either the same residue or residues having similar properties. We have previously stressed the occurrence of functional replacements of evolutionarily conserved residues and suggested that even a slight difference in function conferred by the normal residue is strongly selected for over the course of the evolutionary time scale (Hampsey et al., 1988).

Although highly conserved, the somewhat limited mutational analysis suggests that iso-1-cytochromes *c* can tolerate to various degrees replacements of residues with diverse properties at positions 69−74, 76, and 77 but not at positions 67 and 68; position 75 appears to tolerate restricted classes of residues. Interpretations of replacements at positions 67 and 68, based on the three-dimensional structure of iso-1-cytochrome *c*, have been presented by Hampsey et al. (1988). Ile75 is internally located in the hydrophobic region of the protein (Louie & Brayer, 1990), thus possibly restricting functional replacements to hydrophobic residues.

*Trimethylation of Lys72 in Iso-1-cytochrome c with Single Amino Acid Replacements*. The purified iso-1-cytochromes *c* were digested with *Achromobacter* protease I and the digests were analyzed by reverse phase HPLC (Figure 3).

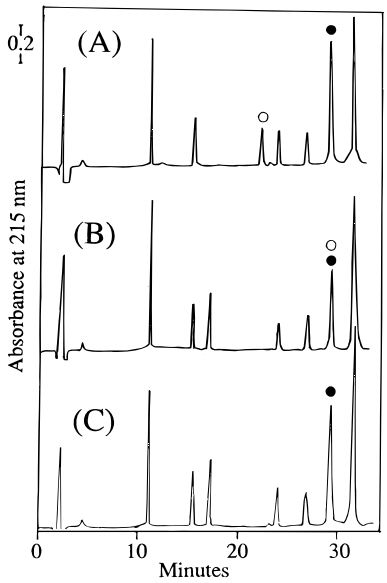


FIGURE 3: Examples of HPLC profiles of peptides obtained by lysylendopeptidase digestion of normal and mutant forms of iso-1-cytochrome *c*. (A) P81L (*CYC1-94-J*); (B) P76E (*CYC1-1072*); (C) normal (*CYC1*$^+$). A total of 5 nmol of each digest was dissolved in 100 $\mu$L of 0.1% trifluoroacetic acid, applied on a $\mu$Bondasphere column, and eluted with a linear gradient as described in the Experimental Procedures. The peptides indicated by ●, encompassing Asn56−Lys73, and the peptides indicated by ○, containing amino acid replacements, were collected and subjected to amino acid compositional analysis. These results were used to establish the percent Tml72.

The peptides corresponding to Asn56−Lys73 were identified by amino acid compositional analysis. The degree of trimethylation of the altered iso-1-cytochromes *c* were calculated based on the Tml content of the Asn56−Lys73 peptides. The amino acid compositions of the Asn56−Lys73 peptides derived from the altered iso-1-cytochromes *c* (data not presented) were used to establish the degree of trimethylation of the altered iso-1-cytochromes *c*, as presented in Table 1 and summarized in Figure 1A. The amino acid replacements were also confirmed with the amino acid compositions. The replacement of Tyr67, Leu68, Thr69, Asn70, Lys73, Pro76, and Gly77 did not affect the degree of trimethylation of Lys72. The P71V and I75Q replacements slightly diminished the Tml content. As expected, the K72R and K72D replacements completely abolished trimethylation of iso-1-cytochrome *c*. Most importantly, there was no trimethyation of Lys72 in the iso-1-cytochromes *c* having Y74E and Y74K replacements. Furthermore, the Y74Q and Y74A replacements resulted in only a partial trimethylation of Lys72. The Y74F iso-1-cytochrome *c* was trimethylated at Lys72 to the same level as the wild-type protein. These results indicate that Lys72 and a nearby aromatic residue are critical for trimethylation of iso-1-cytochrome *c*.

There is marked discrepancy between the degree of trimethylation of Lys72 reported in our study (Table 1), and those reported by Ceesay et al. (1994). The percentages of normal Tml72 in iso-1-cytochromes *c* having the following replacements were deduced from the results presented by Ceesay et al. (1994); 27%, P71V; 65%, K73M; 38%, K73R; 14%, Y74F; 10%, Y74F; and 13%, P71Y K73M. Thus, the values for P71V and Y74F do not correspond in the two studies. This discrepancy could be due to the indirect methods used by Ceesay et al. (1994) for determining the level of Tml72, which was inferred from the level of [methyl-$^3H$]-L-methionine incorporation in Tml.

*Lack of Trimethylation of Lysine Residues at Other Sites*. Because trimethylation of the lysine residue is dependent on the nearby tyrosine residue, we have investigated abnormal sequences -Lys-Any-Tyr- near Lys72 and in the amino-terminal region of the molecule, as shown in Figure 2B and 2C, respectively. The results, summarized in Table 1 and Figure 2, established that, although a sequence -Lys-Any-Tyr- is required for trimethylation, it is not sufficient. Even displacing the sequence one residue to the amino or carboxyl ends does not allow trimethylation. We suggest that an overall conformational structure of the apo-iso-1-cytochrome *c* is required in addition to the properly spaced lysine and an aromatic residues.

Holo-iso-1-cytochrome *c* (Bushnell et al., 1990), as well as other holocytochromes *c*, have well defined structures, whereas apocytochrome *c* exhibits a substantial secondary structure in the presence of high concentrations of salt (Hamada et al., 1993). Furthermore, Dumont et al. (1994) demonstrated that apocytochrome *c* binds strongly to heme *in vitro* and forms a compact structure, similar to holocytochrome *c*, that may represent a natural intermediate. Thus, it is reasonable to assume that apocytochrome *c* can assume a conformational structure required for trimethylation and that this structure is sensitive to amino acid replacements.

We have also confirmed the earlier results of Hickey et al. (1991) that Lys72 of the rat and pigeon cytochromes *c* are not completely trimethylated (Table 1). In addition, strains with multiple gene copies were examined, allowing comparisons of strains having nearly equivalent amounts of cytochrome *c*. We suggest that the incomplete trimethylation of Lys72 in the rat and pigeon cytochromes *c* reflect the slightly different three-dimensional structures of the vertebrate cytochromes *c* compared to the yeast isocytochromes *c*.

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